

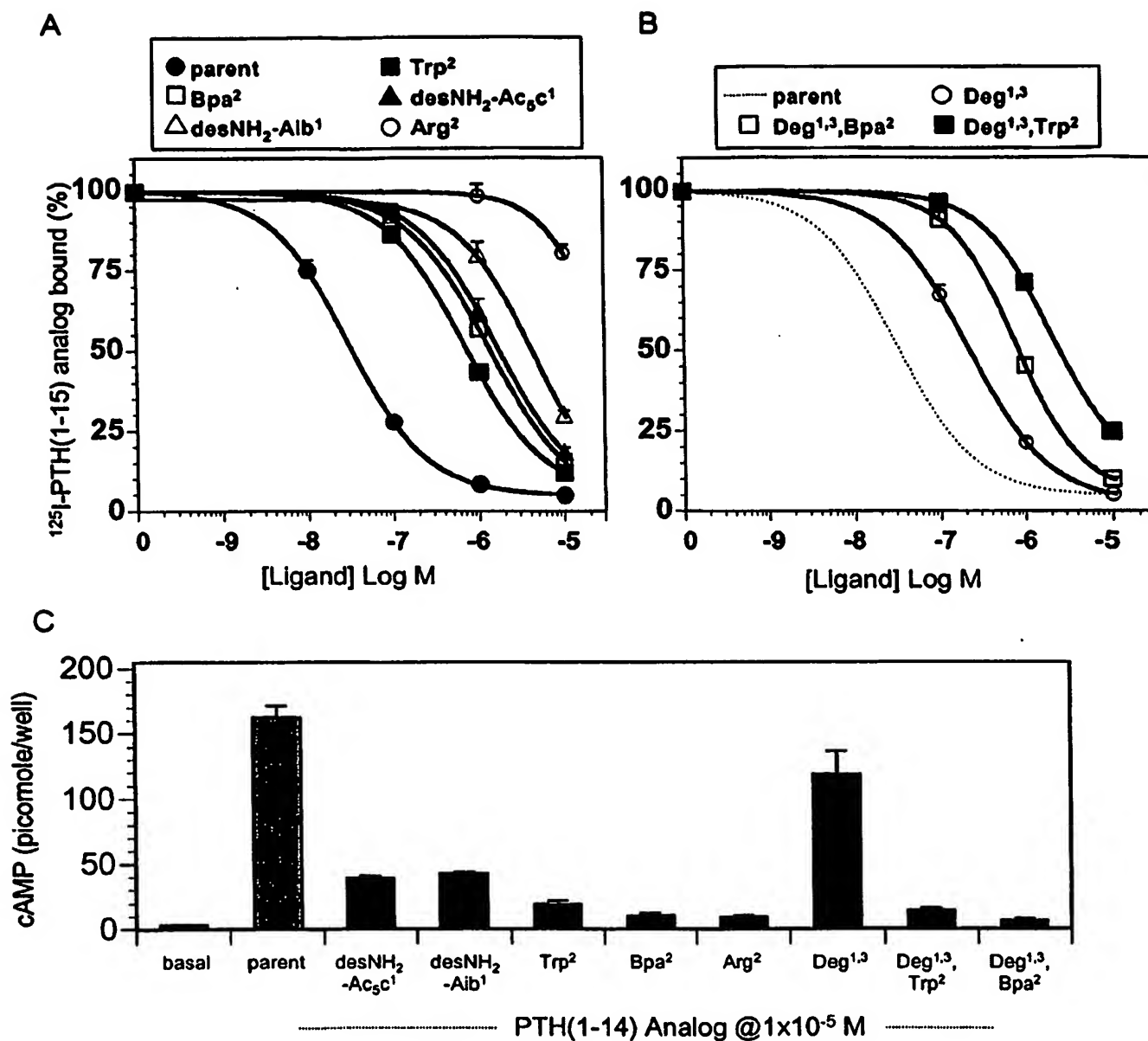
SEQ ID NO.	Peptide	Sequences
	PTH(1-14) peptides	
26	PTH(1-14)NH <sub>2</sub> (native, rat)	Ala-Val-Ser-Glu-Ile-Gln-Leu-Met-His-Asn-Leu-Gly-Lys-His-NH <sub>2</sub>
27	[Ala, <sup>3,12</sup> ,Gln <sup>10</sup> ,Har <sup>11</sup> ,Trp <sup>14</sup> ]PTH(1-14)NH <sub>2</sub>	Ala-Val-Ala-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH <sub>2</sub>
14	[Ac <sub>3</sub> c <sup>1</sup> ,Aib <sup>3</sup> ,Gln <sup>10</sup> ,Har <sup>11</sup> ,Ala <sup>12</sup> ,Trp <sup>14</sup> ]PTH(1-14)NH <sub>2</sub>	Ac <sub>3</sub> c-Val-Aib-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH <sub>2</sub>
15	[desNH <sub>2</sub> -Ac <sub>3</sub> c <sup>1</sup> ,Aib <sup>3</sup> ,Gln <sup>10</sup> ,Har <sup>11</sup> ,Ala <sup>12</sup> ,Trp <sup>14</sup> ]PTH(1-14)NH <sub>2</sub>	(desNH <sub>2</sub> )Ac <sub>3</sub> c-Val-Aib-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH <sub>2</sub>
16	[desNH <sub>2</sub> -Aib <sup>3</sup> ,Aib <sup>3</sup> ,Gln <sup>10</sup> ,Har <sup>11</sup> ,Ala <sup>12</sup> ,Trp <sup>14</sup> ]PTH(1-14)NH <sub>2</sub>	(desNH <sub>2</sub> )Aib-Val-Aib-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH <sub>2</sub>
17	[Ac <sub>3</sub> c <sup>1</sup> ,Trp <sup>2</sup> ,Aib <sup>3</sup> ,Gln <sup>10</sup> ,Har <sup>11</sup> ,Ala <sup>12</sup> ,Trp <sup>14</sup> ]PTH(1-14)NH <sub>2</sub>	Ac <sub>3</sub> c-Trp-Aib-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH <sub>2</sub>
18	[Ac <sub>3</sub> c <sup>1</sup> ,Bpa <sup>2</sup> ,Aib <sup>3</sup> ,Gln <sup>10</sup> ,Har <sup>11</sup> ,Ala <sup>12</sup> ,Trp <sup>14</sup> ]PTH(1-14)NH <sub>2</sub>	Ac <sub>3</sub> c-Bpa-Aib-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH <sub>2</sub>
19	[Ac <sub>3</sub> c <sup>1</sup> ,Arg <sup>2</sup> ,Aib <sup>3</sup> ,Gln <sup>10</sup> ,Har <sup>11</sup> ,Ala <sup>12</sup> ,Trp <sup>14</sup> ]PTH(1-14)NH <sub>2</sub>	Ac <sub>3</sub> c-Arg-Aib-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH <sub>2</sub>
20	[Deg <sup>1,3</sup> ,Gln <sup>10</sup> ,Har <sup>11</sup> ,Ala <sup>12</sup> ,Trp <sup>14</sup> ]PTH(1-14)NH <sub>2</sub>	Deg-Val-Deg-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH <sub>2</sub>
21	[Deg <sup>1,3</sup> ,Trp <sup>2</sup> ,Gln <sup>10</sup> ,Har <sup>11</sup> ,Ala <sup>12</sup> ,Trp <sup>14</sup> ]PTH(1-14)NH <sub>2</sub>	Deg-Trp-Deg-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH <sub>2</sub>
22	[Deg <sup>1,3</sup> ,Bpa <sup>2</sup> ,Gln <sup>10</sup> ,Har <sup>11</sup> ,Ala <sup>12</sup> ,Trp <sup>14</sup> ]PTH(1-14)NH <sub>2</sub>	Deg-Bpa-Deg-Glu-Ile-Gln-Leu-Met-His-Gln-Har-Ala-Lys-Trp-NH <sub>2</sub>
23	[Ac <sub>3</sub> c <sup>1</sup> ,Trp <sup>2</sup> ,Aib <sup>3</sup> ,Nle <sup>8</sup> ,Gln <sup>10</sup> ,Har <sup>11</sup> ,Ala <sup>12</sup> ,Tyr <sup>14</sup> ]PTH(1-14)NH <sub>2</sub>	Ac <sub>3</sub> c-Trp-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-Ala-Lys-Tyr-NH <sub>2</sub>
24	[Ac <sub>3</sub> c <sup>1</sup> ,Bpa <sup>2</sup> ,Aib <sup>3</sup> ,Nle <sup>8</sup> ,Gln <sup>10</sup> ,Har <sup>11</sup> ,Ala <sup>12</sup> ,Tyr <sup>14</sup> ]PTH(1-14)NH <sub>2</sub>	Ac <sub>3</sub> c-Bpa-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-Ala-Lys-Tyr-NH <sub>2</sub>
25	[Deg <sup>1,3</sup> ,Bpa <sup>2</sup> ,Nle <sup>8</sup> ,Gln <sup>10</sup> ,Har <sup>11</sup> ,Ala <sup>12</sup> ,Trp <sup>14</sup> ,Arg <sup>19</sup> ,Tyr <sup>21</sup> ]PTH(1-21)NH <sub>2</sub>	Deg-Bpa-Deg-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-Ala-Lys-Trp-Leu-Ala-Ser-Val-Arg-Arg-Tyr-NH <sub>2</sub>
	N-truncated peptides	
28	[Aib <sup>3</sup> ,Nle <sup>8</sup> ,Gln <sup>10</sup> ,Har <sup>11</sup> ,Ala <sup>12</sup> ,Trp <sup>14</sup> ,Arg <sup>19</sup> ,Tyr <sup>21</sup> ]PTH(3-21)NH <sub>2</sub>	Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-Ala-Lys-Trp-Leu-Ala-Ser-Val-Arg-Arg-Tyr-NH <sub>2</sub>
29	[Ile <sup>5</sup> ,Trp <sup>23</sup> ,Tyr <sup>36</sup> ]PTHrP(5-36)NH <sub>2</sub>	Ile-Gln-Leu-Leu-His-Asp-Lys-Gly-Lys-Ser-Ile-Gln-Asp-Leu-Arg-Arg-Arg-Phe-Phe-Leu-His-His-Leu-Ile-Ala-Glu-Ile-His-Thr-Ala-Glu-Tyr*-NH <sub>2</sub>
31	[Ile <sup>5</sup> ,Leu <sup>11</sup> ,D-Trp <sup>12</sup> ,Trp <sup>23</sup> ,Tyr <sup>36</sup> ]PTHrP(5-36)NH <sub>2</sub>	Ile-Gln-Leu-Leu-His-Asp-Leu-DTrp-Lys-Ser-Ile-Gln-Asp-Leu-Arg-Arg-Arg-Phe-Phe-Leu-His-His-Leu-Ile-Ala-Glu-Ile-His-Thr-Ala-Glu-Tyr*-NH <sub>2</sub>
	<sup>125</sup> I-PTH tracer radioligand	
32	[Aib <sup>1,3</sup> ,Nle <sup>8</sup> ,Gln <sup>10</sup> ,Har <sup>11</sup> ,Ala <sup>12</sup> ,Trp <sup>14</sup> ,Tyr <sup>15</sup> ]PTH(1-15)NH <sub>2</sub>	Aib-Val-Aib-Glu-Ile-Gln-Leu-Nle-His-Gln-Har-Ala-Lys-Trp-Tyr*-NH <sub>2</sub>

Figure 1

	Peptides	IC <sub>50</sub>		
	PTH(1-14) peptides		nM	n
##	parent	30	±7	3
##	desNH <sub>2</sub> -Alb <sup>1</sup>	4,500	±700	4
##	desNH <sub>2</sub> -AC <sub>5</sub> C <sup>1</sup>	1,800	±100	4
##	Arg <sup>2</sup>	25,000	±2,000	4
##	Trp <sup>2</sup>	770	±110	4
##	Bpa <sup>2</sup>	1,400	±200	4
##	Deg <sup>1,3</sup>	230	±50	3
##	Deg <sup>1,3</sup> ,Trp <sup>2</sup>	2,700	±300	3
##	Deg <sup>1,3</sup> ,Bpa <sup>2</sup>	840	±110	3
	Other peptides			
##	rPTH(1-34)	4.8	±0.8	3
##	PTHrP(5-36)	5.5	±1.0	3
##	[Aib3,M]PTH(3-21)	750	±90	3
##	[Aib1,3,M]PTH(1-21)	18	±4	3

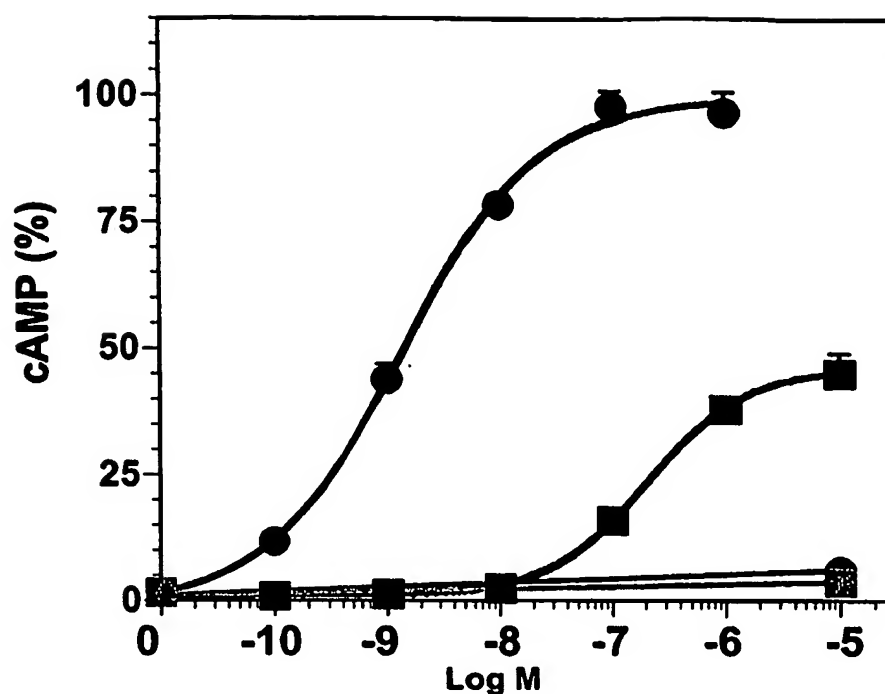
Figure 2

Figure 3



**Functional Responses in HKRK-B28 Cells.** Binding (A and B) and cAMP agonism/partial agonism assays (C) were performed in HKRK-B28 cells. The parent peptide was [AC5C1,Aib3,Gln10,Har11,Ala12,Trp14]PTH(1-14)NH<sub>2</sub> and derivatives thereof were substituted at positions 1, 2 and/or 3, as indicated. Binding assays (4h @ 15°C) were performed with <sup>125</sup>I-[Alb1,3,Nle8,Gln10,Har11,Ala12,Trp14,Tyr15]PTH(1-15)NH<sub>2</sub> tracer. cAMP assays were performed at RT for 30 min. Relative to the parent, the substituted analogs lack appreciable agonist activity.

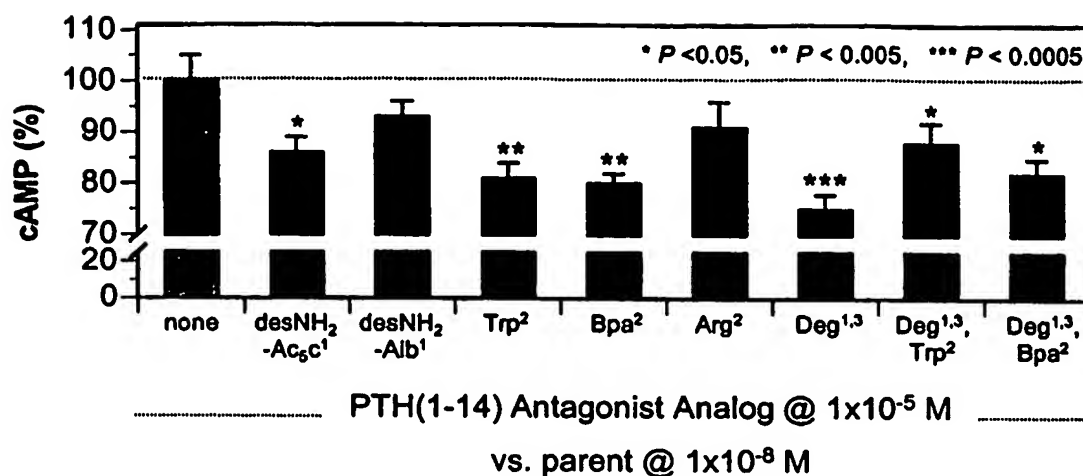
Figure 4



	<u>EC<sub>50</sub> nM</u>
● parent	1.7±0.3
■ Deg <sup>1,3</sup>	220±50
● Bpa <sup>2</sup>	-----
■ Deg <sup>1,3</sup> ,Bpa <sup>2</sup>	-----

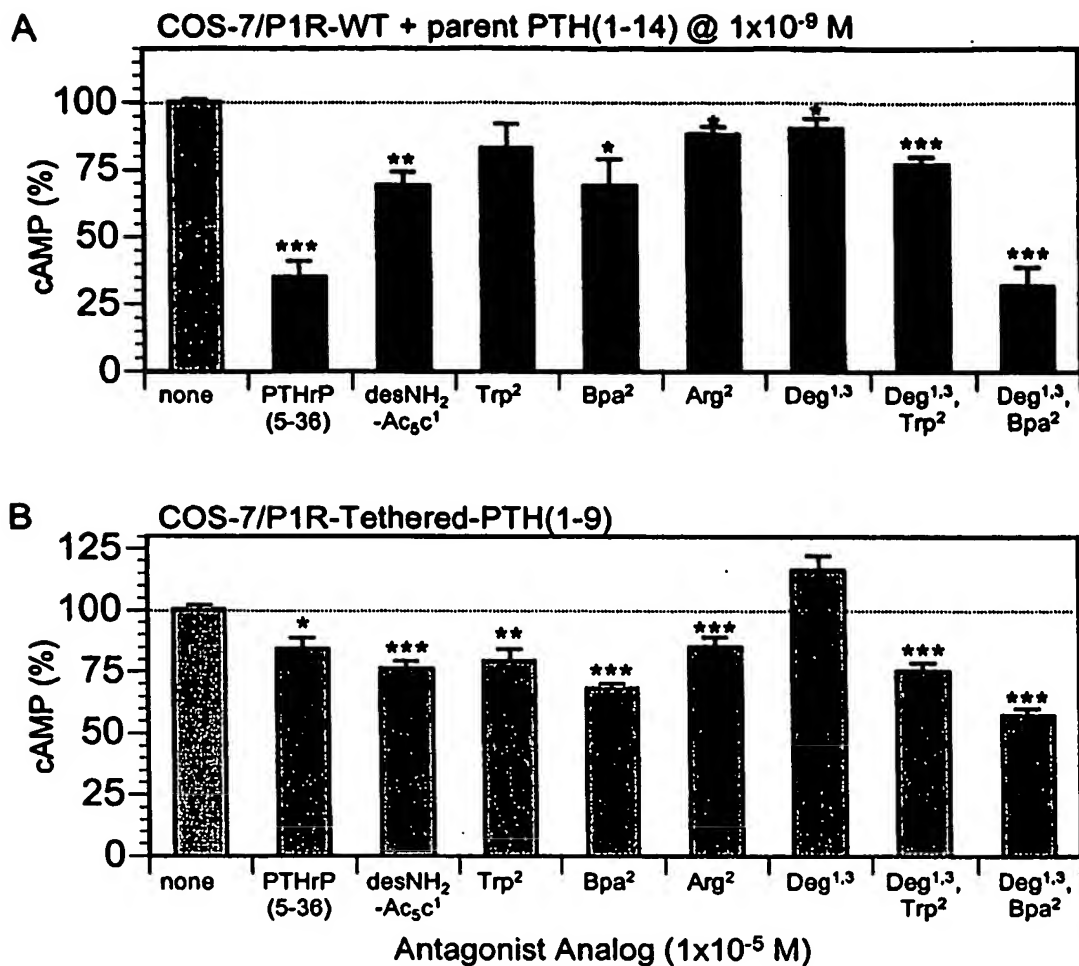
**cAMP Responses in HKRK-B28 Cells.** The parent peptide, [AC5C1,Aib3,Gln10,Har11,Ala12,Trp14]PTH(1-14)NH<sub>2</sub>, and derivatives thereof substituted at positions 1, 2 and/or 3, as indicated, were assayed for cAMP agonist responses in HKRK-B28 cells. The parent peptide functions as a fully potent and efficacious agonist, the Deg1,3-substituted analog is a partial agonist, and the Bpa2-substituted analogs lack agonist activity.

Figure 5



**Antagonism Assays in HK-RK-B28 Cells.** cAMP antagonism assays were performed in HKRK-B28 cells. Cells were treated with the J domain-selective agonist, [AC5C1,Aib3,Gln10,Har11,Ala12,Trp14]PTH(1-14)NH<sub>2</sub> (parent) at 10 nM, either alone (none) or with a candidate antagonist peptide (10  $\mu$ M), which was a derivative of the parent PTH(1-14) peptide substituted at positions 1, 2 and/or 3, as indicated. Asterisks indicate significant reductions in cAMP levels, as compared to cells not treated with antagonist (none).

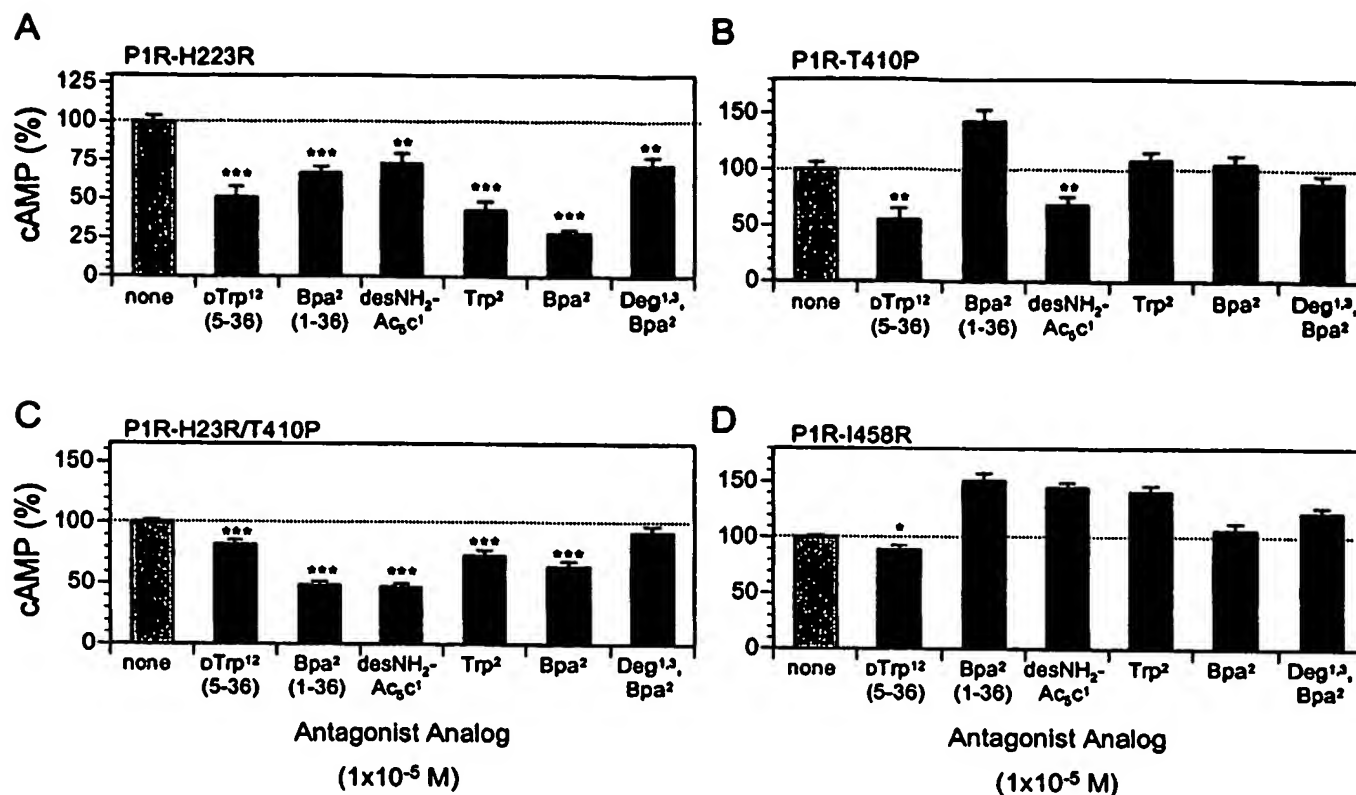
Figure 6



\*  $P < 0.05$ , \*\*  $P < 0.005$ , \*\*\*  $P < 0.0005$

**Antagonism Assays in COS-7 Cells.** cAMP antagonism assays were performed in COS-7 cells transfected with the wild-type P1R (A), or a constitutively active P1R derivative having the first 9 residues of PTH tethered to TM1 of the P1R and in place of the P1R N-terminal domain (inset, B). In A, cells were treated with the J domain-selective agonist, [AC5C1,Aib3,Gln10,Har11,Ala12,Trp14]PTH(1-14)NH<sub>2</sub> (parent) at 1 nM, alone (none) or with a candidate antagonist peptide (10  $\mu$ M), which was a derivative of the parent PTH(1-14) peptide substituted at positions 1, 2 and/or 3, as indicated, or [I5,W23,Y36]PTHrP(5-36) analog. Asterisks indicate significant reductions in cAMP levels, as compared to cells not treated with antagonist (none).

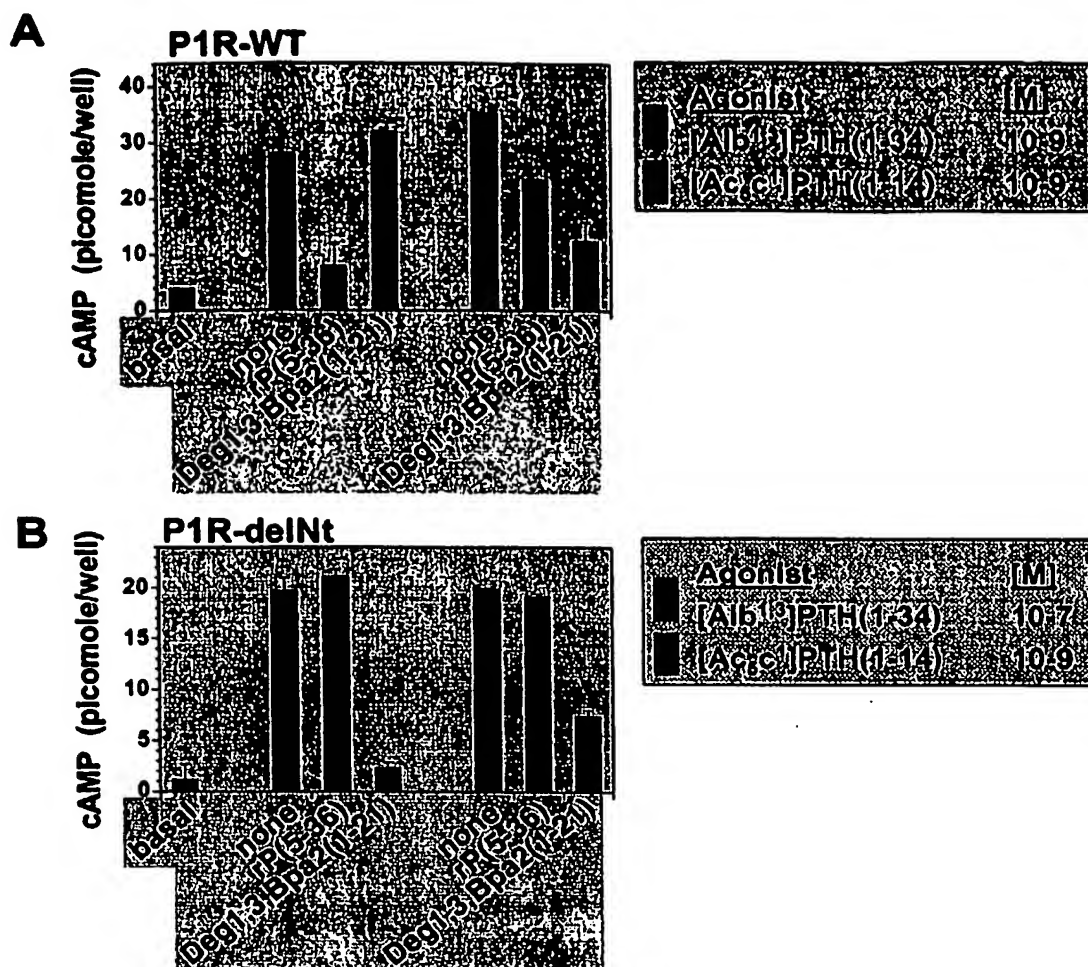
Figure 7



\*  $P < 0.05$ , \*\*  $P < 0.005$ , \*\*\*  $P < 0.0005$

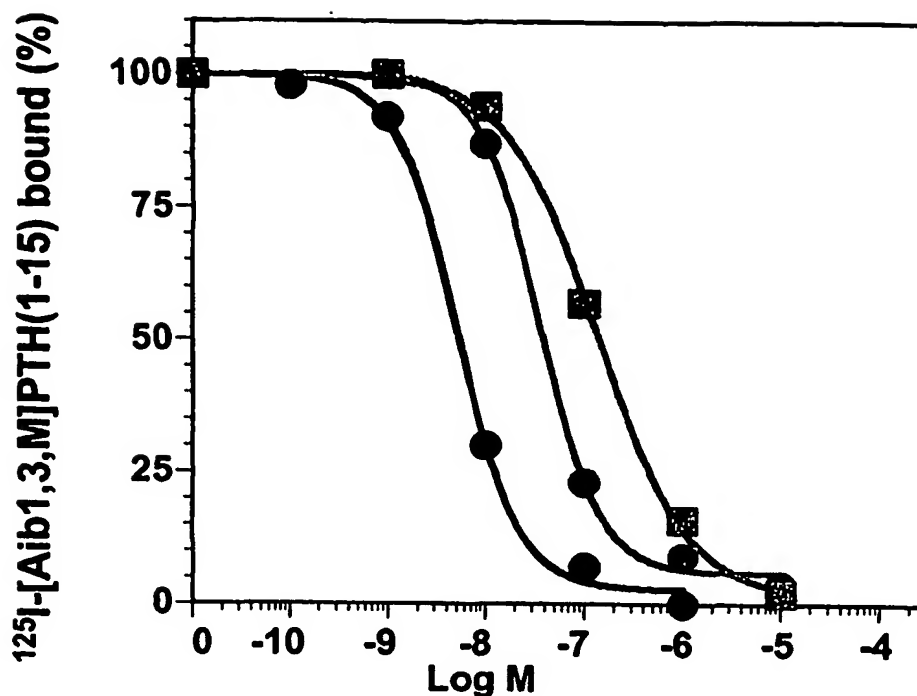
**Inverse Agonist Responses in COS-7 Cells.** COS-7 cells were transfected with the constitutively active P1Rs: P1R-H223R (A), P1R-T410P (B), P1R-H223R/T410P (C), or P1R-I458R (D) and then were incubated (30 min@R.T.) either in the absence of peptide (none) or in the presence of the indicated antagonist/inverse agonist peptide (10  $\mu$ M), and cAMP was measured by RIA. Asterisks indicate significant reductions in cAMP levels, compared to untreated cells (none).

Figure 8



**"N" versus "J" Domain selectivity of P1R Antagonists in COS-7 Cells.** cAMP antagonism assays were performed in COS-7 cells transfected with the wild-type P1R (A), or a P1R derivative (P1R-delNt) having most (residues 24-181) of the P1R N domain deleted (B). Cells were treated with the agonist [Aib1,3,Tyr34]hPTH(1-34)NH<sub>2</sub> ([Aib1,3]PTH(1-34)), which utilizes both N and J domains for affinity/potency, or with [AC5C1,Aib3,Gln10,His11,Ala12,Trp14]PTH(1-14)NH<sub>2</sub> ([Ac5c1]PTH(1-14)), which uses only the J domain for affinity/potency, at the concentrations indicated in the key, so as to elicit half-maximum cAMP responses in the absence of antagonist (none). The analogs PTHrP(5-36) and Deg1,3,Bpa2-PTH(1-21) were added at  $1 \times 10^{-5}$  M, as indicated. On the WT receptor, PTHrP(5-36) antagonizes PTH(1-34) analog more effectively than does Deg1,3,Bpa2-PTH(1-21), but the PTH(1-21) analog antagonizes PTH(1-14), more effectively than does PTHrP(5-36). On P1R-delNt, Deg1,3,Bpa2-PTH(1-21) antagonizes either agonist, whereas PTHrP(5-36) lacks antagonist capability. Thus, PTHrP(5-36) is an N domain-selective antagonist, whereas Deg1,3,Bpa2-PTH(1-21) is a J domain-selective antagonist. The analog Deg1,3,Bpa2-PTH(1-14) behaved similarly in these assays to Deg1,3,Bpa2-PTH(1-21).

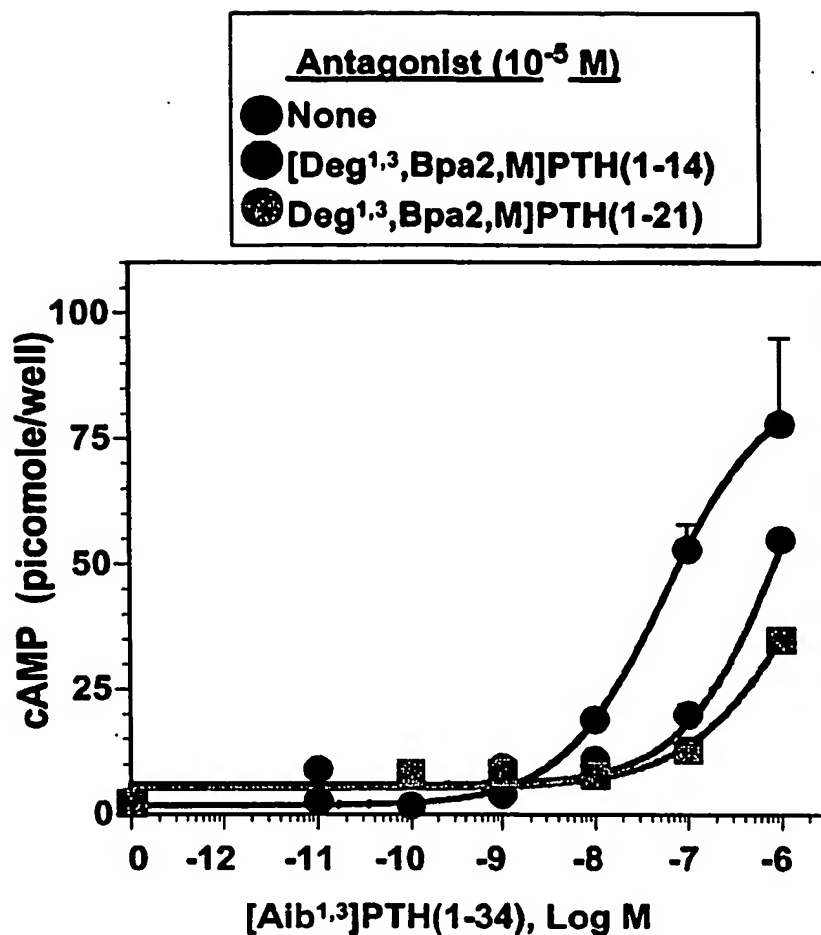
Figure 9



	<u>IC<sub>50</sub>(nM)</u>
● PTH(1-34)	2
● PTHrP(5-36)	50
■ [Deg <sup>1,3</sup> ,Bpa <sup>2</sup> ,Y <sup>15</sup> ,M]PTH(1-21)	150

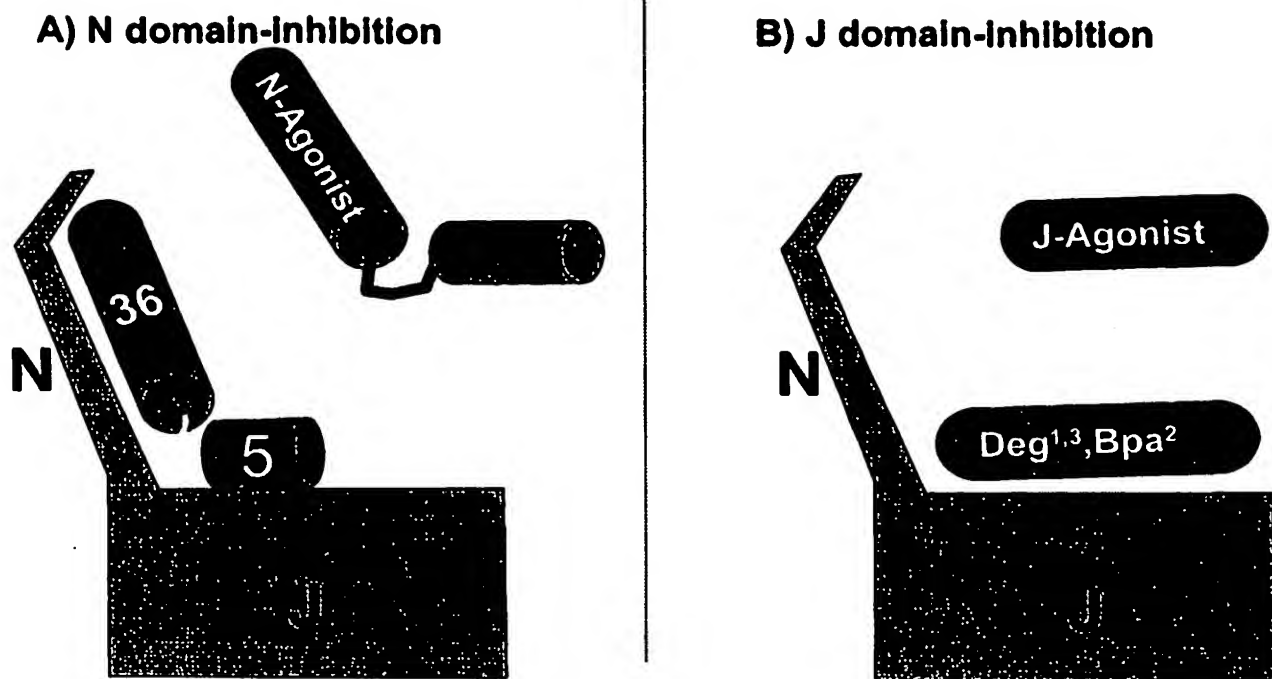
**Competition Binding Assays in HKRK-B7 Cells.** Binding assays were performed in HKRK-B7 cells, which express the wild-type hP1R, using <sup>125</sup>I-[Aib1,3,Nle8,Gln10,Har11,Ala12,Trp14,Tyr15]PTH(1-15)NH<sub>2</sub> as a tracer radioligand and the indicated unlabeled peptides as competitors. PTH(1-34) is [Tyr34]hPTH(1-34)NH<sub>2</sub>.

Figure 10



**Competitive Antagonism on P1R-deInt.** COS-7 cells transfected with P1R-deInt were stimulated with varying concentrations of the agonist [Aib1,3,Tyr34]hPTH(1-34)NH<sub>2</sub> ([Aib1,3]PTH(1-34)), either in the absence of antagonist (green circles) or in the presence of an antagonist, [Deg1,3,Bpa2,M]PTH(1-14) (red circles) or [Deg1,3,Bpa2,M]PTH(1-21) (yellow squares) each at  $1 \times 10^{-5}$  M, as indicated in the figure key. Each antagonist causes a parallel, right-ward shift in the agonist dose-response curve, which is consistent with a competitive mechanism of inhibition.

Figure 11



**Two Modes of Competitive Inhibition at the P1R.** Two modes of antagonism are now recognized at the P1R. N domain inhibition (A) is utilized by most conventional P1R antagonists, such as PTHrP(5-36) and PTHrP(7-34) analogs, and is based on the derivation of binding energy primarily from interactions between the (21-34) region of the ligand and the P1R N domain. This mechanism is effective for inhibition of N-domain-dependent agonists, such as PTH(1-34), but not for N domain-independent agonists, such as PTH(1-14). J domain inhibition (B) is utilized by the novel analogs described herein, and is based on the derivation of binding energy primarily or wholly from interactions between the (1-20) region of the ligand and the J domain of the P1R. This mechanism is effective for inhibition of J-domain-dependent agonists, such as PTH(1-14) analogs, but not for N domain-dependent agonists, such as PTH(1-34). A J domain-selective antagonists would be useful for characterizing small-molecules that act as PTH mimetics, since such molecules are likely to bind to the J domain.

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